

### **AMENDMENT TO THE CLAIMS:**

This listing of claims will replace all prior version, and listings, of claims in the application:

### **LISTING OF CLAIMS:**

1. (Canceled)
2. (Original) A Process for the isolation of p-benzosemiquinone of formula 1, a major harmful oxidant from cigarette smoke responsible for the oxidative damage of proteins and DNA, the said process further comprising
  - (a) passing the whole cigarette smoke collected from conventional filter tipped cigarette having a tar content of 20 -30 mg per cigarette into 30-60 mM potassium buffer at pH 7.4 –7.8, filtering the above solution through 0.45 µm Millipore filter, adjusting the pH of the filtrate ranging between 7.4 to 7.6 by adding NaOH solution to obtain the desired cigarette smoke solution (cs solution);
  - (b) extracting the above said cs solution thrice with equal volume of methylene chloride, discarding the lower methylene chloride layer and collecting the upper yellow coloured aqueous layer termed as aqueous extract of cigarette smoke;
  - (c) extracting the above said aqueous layer of cigarette smoke twice with equal volume of water saturated n-butanol, lyophilizing the pooled yellow butanol extract in a Lyolab lyophilizer at a temperature ranging between –50 °C to –60 °C under vacuum followed by extraction of the lyophilized material twice with HPLC grade acetone and drying the acetone solution under vacuum and dissolving the said acetone extract with HPLC grade methanol;
  - (d) subjecting the above said methanol solution to band TLC using non-fluorescent silica plates, developing the said silica plates using a mixture of toluene and ethyl acetate in a ratio of 80:20, taking out the plate and drying

at about 25 ° C to 30 ° C using a drier, cutting small strips containing the developed material from both sides of the plates and keeping them in an iodine chamber for the location of the band corresponding to Rf 0.26 , scraping the band and extracting the band material with HPLC grade acetone followed by collection of the acetone layer and drying it under vacuum;

- (e) dissolving the above said acetone extract which appeared as pale yellow needles by adding equal volume of milli Q water , extracting the aqueous solution with equal volume of HPLC grade water saturated n-butanol followed by drying the upper n-butanol layer in small glass tubes under vacuum to obtain the major cs oxidant with a purity of 98-99% and yield of 18-22 µg per cigarette; and
- (f) purifying the above said cs oxidant as obtained in step e by dissolving it in a mobile solvent comprising a mixture of methylene chloride and methanol in a ratio of 90:10(v/v) and injecting it in a HPLC instrument with a normal phase 25 cm silica column using a uv detector at 294 nm at a flow rate of 0.5 ml/min, at a temperature of about 25°C, at a pressure of about 29 kgf/cm<sup>2</sup> and collecting the effluent which appears as a single peak at a retention time of 8.808 min with a purity of 100 % and yield of 8.4% of the total cs oxidant present in the parent cs solution.
- (g) dissolving the above said acetone extract which appeared as pale yellow needles by adding equal volume of milli Q water, extracting the resultant aqueous solution with equal volume of HPLC grade water saturated n-butanol followed by drying upper n-butanol layer in small glass tubes under vacuum to obtain the major cigarette smoke (cs) oxidant with a purity of 98-99% and yield of about 18-22 µg per cigarette, and
- (h) purifying the above said cs oxidant as obtained in step (g) by dissolving it in a mobile solvent comprising a mixture of methylene chloride and methanol in a ratio of 90:10 (v/v) and injecting it in a HPLC instrument with a normal phase 25 cm silica column using a uv detector at 294 nm at a flow rate of 0.5 ml/min, at a temperature of about 25°C and at a pressure of about 29 kgf/cm<sup>2</sup> followed by collecting the effluent which appears as a single peak at a retention time of 8.808 min with a purity of 100 % and yield of 8.4% of the total cs oxidant present in the parent tar solution.

3-4. (Canceled)

5. (Original) A Process for the quantitative determination of p-benzosemiquinone of formula 1, a major harmful oxidant from cigarette smoke responsible for the oxidative damage of proteins and DNA, the said process further comprising

- (a) passing the whole cigarette smoke collected from conventional filter tipped cigarette having a tar content of 20 -30 mg per cigarette into 30-60 mM potassium buffer at pH 7.4 -7.8, filtering the above solution through 0.45  $\mu$ m Millipore filter, adjusting the pH of the filtrate ranging between 7.4 to 7.6 by adding NaOH solution to obtain the desired cigarette smoke solution (cs solution),
- (b) extracting the above said cs solution thrice with equal volume of methylene chloride, discarding the lower methylene chloride layer and collecting the upper yellow colored aqueous layer termed as aqueous extract of cigarette smoke,
- (c) extracting the above said aqueous layer of cigarette smoke twice with equal volume of water saturated n-butanol, lyophilizing the pooled yellow butanol extract in a Lyolab lyophilizer at a temperature ranging between  $-50^{\circ}\text{C}$  to  $-60^{\circ}\text{C}$  under vacuum followed by extraction of the lyophilized material twice with HPLC grade acetone and drying the acetone solution under vacuum and dissolving the said acetone extract with HPLC grade methanol,
- (d) subjecting the above said methanol solution to band TLC using non-fluorescent silica plates, developing the said silica plates using a mixture of toluene and ethyl acetate in a ratio of 80:20, taking out the plate and drying at about  $25^{\circ}\text{C}$  to  $30^{\circ}\text{C}$  using a drier, cutting small strips containing the developed material from both sides of the plates and keeping them in an iodine chamber for the location of the band corresponding to  $R_f$  0.26, scraping the band and extracting the band material with HPLC grade acetone followed by collection of the acetone layer and drying it under vacuum,

- (e) dissolving the above said acetone extract which appeared as pale yellow needles by adding equal volume of milli Q water, extracting the aqueous solution with equal volume of HPLC grade water saturated n-butanol followed by drying the upper n-butanol layer in small glass tubes under vacuum to obtain the major cs oxidant with a purity of 98-99% and yield of 18-22 µg per cigarette, and
- (f) purifying the above said cs oxidant as obtained in step e by dissolving it in a mobile solvent comprising a mixture of methylene chloride and methanol in a ratio of 90:10(v/v) and injecting it in a HPLC instrument with a normal phase 25 cm silica column using a uv detector at 249 nm at a flow rate of 0.5 ml/min, at a temperature of about 25°C, at a pressure of about 29 kgf/cm<sup>2</sup> and collecting the effluent which appears as single peak at a retention time of 8.808 min with a purity of 100% and yield of 8.4% of the total cs oxidant present in the parent cs solution.

6-38. (Canceled)

39. (New) A process as claimed in claim 2, wherein said isolated pure cigarette smoke (cs) oxidant has the following properties:
- (a) when crystallized from acetone solution appears as small needle shaped faint yellow coloured crystals having pungent smell, similar to that of rancid butterfat,
  - (b) UV absorption maxima in methanol solution are at 293.4 nm and 223.0 nm and in aqueous solution are at 288nm and 221nm, respectively,
  - (c) on excitation at 293 nm in methanol solution the observed emission maxima are at 329.6 nm and 651.4 nm and on excitation at 224 nm, the observed emission maxima are at 329.6 nm and 652.6 nm, respectively,
  - (d) when excitation scanning is monitored keeping the emission at 330 nm, the observed excitation maxima are at 228.2 nm and 293.8 nm and when the emission is kept at 651 nm and excitation scanning is monitored, the observed excitation maxima are at 229.2 nm and 294.8 nm, respectively,

- (e) highly soluble in methanol, ethanol, acetone, n-butanol, fairly soluble in water, sparingly soluble in methylene chloride, di-ethyl ether, chloroform and insoluble in benzene and petroleum ether,
- (f) the compound loses its oxidizing potency in acidic pH ranging between 4 to 5 and on keeping the solution at alkaline pH ranging between 9 to 10, the compound gradually turns brown, at pH 10 and above there is instantaneous darkening with loss of both activity and aromaticity as evidenced by UV spectroscopy,
- (g) the half-life of the oxidant, when stored in the solid state at a temperature ranging between 25 °C to 30 °C under darkness is about 48 hours as determined by its oxidative potency, but in solution of 50 mM potassium phosphate buffer, pH 7.4 at 25°C to 30°C the half life is about 1 hour 30 min,
- (h) reduces ferricytochrome c and ferric chloride,
- (i) oxidizes ascorbic acid, proteins and DNA, and
- (j) the melting point is 162°C.

40. (New) A process as claimed in claim 2, wherein p-benzosemiquinone present in solution is quantitatively assayed by HPLC with a UV detector using a 25 cm reverse phase ODS column and using a mixture of water and methanol (95: 5 v/v) as a mobile phase, at a wave length of 288nm, flow rate of 0.8 ml/min, at a temperature of about 25°C and at a pressure of about 147 Kg/cm<sup>2</sup> and having a retention time of 13.46 min.